

1                                   **"LASER DIODE-EXCITED**  
2                                   **BIOLOGICAL PARTICLE DETECTION SYSTEM"**

3  
4                                   FIELD OF THE INVENTION

5                                   The present invention is related to process and apparatus for detecting  
6                                   the presence of biological agents utilizing stimulation and detection of fluorescence  
7                                   therefrom.

8  
9                                   BACKGROUND OF THE INVENTION

10                                  There is a recognized need for the detection of undesirable  
11                                  concentrations of potentially harmful airborne bacteria in health care environments,  
12                                  laboratories and in warfare conditions. To date, equipment used for such purposes  
13                                  have been unreliable and expensive to build and operate. Further, the associated  
14                                  support requirements have been very high, both in power requirements and  
15                                  maintenance. Accordingly, to date, their use has been restricted to industrial and  
16                                  governmental use. The service industry or the homeowner, concerned about  
17                                  residential and environmental monitoring, has not had practical access to such  
18                                  equipment.

19                                  Biological aerosols, mostly harmless, are indigenous to many  
20                                  environments, including homes. Microorganisms are naturally aerosolized in the  
21                                  atmosphere, often becoming a biological burden to downwind communities. For  
22                                  example, municipal wastewater aeration tanks can produce coliform bacteria, and  
23                                  washing of dairy processing plants can produce a persistent biological aerosol. In  
24                                  the hog industry, diffusion modeling has shown that aerosolized microorganisms  
25                                  could spread infection over a 150 km<sup>2</sup> area among swine herds. Indoor

1 microbiological air quality in homes and offices has demonstrated the prevalence of  
 2 bacteria and fungi, for example bacterial numbers can range upwards to 800 viable  
 3 particles/m<sup>3</sup> in homes and up to 500 in offices. Typical values of fungi are about half  
 4 as abundant. Fairly similar findings have been reported as recently as 1998 by the  
 5 US Environment Protection Agency which has recently taken an interest in  
 6 measuring indoor and outdoor bio-logical aerosol quality.

7               Processes for the detection of airborne particles typically comprise  
 8 sizing and characterizing the particles as biological. Such characterization of  
 9 particles as being biological can be accomplished by detecting a specific biomolecule  
 10 present only in bio-viable particles. Most living cells contain a coenzyme or  
 11 biomolecule, nicotinamide adenine dinucleotide phosphate (NADP). NADP is a  
 12 biomolecule which is essential for cellular metabolism as an electron or hydrogen  
 13 acceptor and is therefore an essential constituent in most biological processes to be  
 14 viable. NADP is the oxidized form of NAD(P)H or nicotinamide adenine dinucleotide  
 15 hydrogen (NADH). It is known that NADH can be excited with ultraviolet light and  
 16 will fluoresce. The fluorescence excitation and emission wavelengths of NADH are  
 17 well separated, which facilitates detection. The excitation wavelength of NADH is  
 18 centered at 340 nm in the near ultraviolet spectrum, and its fluorescent emission  
 19 wavelength extends from 400 to 540nm.

20               Apparatus are known for implementing excitation and detection of  
 21 fluorescence of NADP in discrete particles. A fluorescent aerodynamic particle  
 22 sizing (FLAPS) apparatus is disclosed in US Patents 5,701,012 and 5,895,922  
 23 issued to Dr. J. Ho, the entirety of both patents being included herein by reference.  
 24 The Dr. Ho disclosed the use of HeCd and tripled frequency YAG lasers, which emit  
 25 a laser light beams having a wavelength in the range of 340 – 360 nm, ideal for the

1 excitation of NADH. Additional aspects addressed by Dr. Ho include combining the  
2 laser excitation and detection technology with an aerodynamic particle sizer which  
3 implements converging airstreams for separating particles for analysis. Accordingly,  
4 the FLAPS apparatus comprises an aerodynamic particle sizer, the ultraviolet laser  
5 and a photomultiplier tube (PMT) for detecting fluorescence from particles.  
6 Focusing optics direct the laser to contact each passing particle. A further aspect  
7 addressed by Dr. Ho was to implement sequential lasers across the particle's path  
8 for establishing the speed of the particle in the separating airstream, resulting in  
9 enhanced correlation with biological viability.

10 The lasers used in the prior art FLAPS apparatus add significantly to  
11 its complexity. For instance, the 100 cm long, 30 mW, cooled 325 nm Helium-  
12 Cadmium (HeCd) laser and focusing optics are large, heavy, and power intensive.  
13 The disclosed system weighs in at about 90 kg and consumes 800 watts of electrical  
14 power. The HeCd laser, power supply, and the particle sizing 633 nm HeNe lasers  
15 are all mounted to a 60 by 150 centimeter optics table and enclosed with a sheet  
16 metal cover to give the system a height of about 30 cm. In order to operate this  
17 apparatus remote from an industrial site requires the addition of a power generator  
18 and a technician to ensure minimal interruption between failures.

19 To date, the FLAPS apparatus has been limited to the use of an  
20 expensive, and unreliable, light sources such as the conventional HeCd or YAG  
21 lasers described above to provide the wavelengths suitable to excite NADH for  
22 signaling the detection of viable biomolecules and which was known to be suitable  
23 also to detect NADH even in spores.

24 The above prior references identify and set forth the concerns about  
25 the detection of certain hard-to-assess biological particles such as anthrax,

1 particularly in the spore form. Questions were resolved, whether there was sufficient  
2 intrinsically fluorescing biological matter (NADH) to enable one to establish whether  
3 the particle was viable (biologically alive), and thus potentially hazardous. The  
4 30mW HeCd laser disclosed in the FLAPS apparatus was deemed successful for  
5 stimulating measurable fluorescence of NADH from spores.

6               However, as stated, the HeCd laser is expensive, unreliable and  
7 requires laser cooling and a significant power source. This severely limits its  
8 usability in critically hazardous, mobile and portable operations.

9               There was therefore a demonstrated need for a more practical, more  
10 portable and economical approach to biomolecule detection. In improving the  
11 apparatus by applying a new type of laser, the applicant introduced significant  
12 uncertainty in its ability to reliably distinguish viable from non-viable particles. The  
13 laser has different operating properties and characteristics including its power output  
14 and its light wavelength; either of which could limit the apparatus's ability to detect  
15 viable particles. This task ultimately required significant effort and expense to  
16 overcome the specific uncertainty about the ability of such improvements to provide  
17 reliable and repeatable results.

## SUMMARY OF THE INVENTION

Apparatus is provided for identifying viable biomolecules in a stream. The apparatus is an improvement over related instruments, the advantages including achievement of substantial reductions in cost and power consumption which enables widening application of the technology from large industrial, governmental and military applications to also include smaller commercial and residential use. Implementation of the concepts described herein have expanded our knowledge into heretofore unknown response of biomolecules other than NADH.

In the course of improving detection apparatus, the applicant has also discovered that a new range of biomolecules can be used which are indicative of particle bio-viability. Further, in expanding this range, new apparatus having improved economies and efficiencies have also been discovered while disadvantages including complexity and high power demands of the prior art apparatus are avoided.

Simply, in a preferred aspect, a laser diode is provided and applied as the excitation source. To date, laser diodes are not available at the wavelengths known to be most suited to excite the known reactive biomolecule NADH; however, they are currently available at slightly longer wavelengths. Heretofore, it has not been confirmed, whether biomolecules indicative of bio-viability exist (other than NADH) which provide similar fluorescence characteristics under laser light at other wavelengths or which are discernable at lower light emission power. It had not been confirmed whether biomolecules such as flavinoids, believed to be excited at wavelengths longer than that used to excite NADH, are even present at all in hazardous particles like spores, or are even available in sufficient quantities to be excited by laser light. Neither has it been determined that such longer wavelengths

1 would even be suitable for exciting any biomolecules, such as flavinoids, so as to  
2 produce measurable fluorescence.

3 Surprisingly, applicant has determined that viable particles, including  
4 spores, can be excited to fluoresce using a laser diode operating with power output  
5 and power requirements as low as about 8 -15 mWatts. Conventional apparatus  
6 using a shorter wavelength He-Cd laser may also operate at relatively low power  
7 output levels, such as about 30mW, however the practical and overall power  
8 requirements to operate the laser are many orders of magnitude greater (say 800  
9 Watts) when the operation of necessary ancillary equipment such as laser cooling  
10 systems are included. Further, diode lasers are distinguished from conventional gas  
11 or solid state lasers by their ability to be pumped directly by an electrical current  
12 which results in efficient operation, approaching power conversion efficiencies of  
13 50%. The gas and solid state lasers, which generally pumped by plasma excitations  
14 or an incoherent optical flash lamp source respectively, exhibit efficiencies which are  
15 more in the order of 1%. Another difference between diode and other lasers is their  
16 physical size; wherein gas and diode pumped solid state lasers are typically tens of  
17 centimeters in length, laser diode assemblies are generally about the size of the  
18 grain of salt and are only bulked up slightly (to about one centimeter) mostly for the  
19 purpose of handling and mounting purposes.

20 Therefore, in a broad aspect of the invention an improved apparatus is  
21 provided for identifying the existence of biologically viable particles within a particle  
22 population containing a mixture of biologically viable and biologically inert particles.  
23 The improved apparatus comprises: a solid state excitation source wherein said  
24 source is a laser diode for emitting a light beam being directed to contact particles of  
25 the particle population and having a wavelength from about 320nm to 500nm which

1 is operative to excite biomolecules contained therein to produce fluorescence; a  
2 photon counter for measuring the intensity of fluorescence emitted from each  
3 contacted particle and producing a signal indicative thereof; and a microprocessor  
4 for comparing each contacted particle's fluorescent intensity signal against  
5 predetermined criteria and establishing whether that particle is a biologically viable  
6 particle or an inert particle.

7 Preferably, the improvement is applied in combination with particle  
8 discretization or segregation apparatus and more preferably in combination with  
9 particle sizing apparatus for improving the identification of the biomolecules as  
10 respirable and hazardous.

#### 11 BRIEF DESCRIPTION OF THE DRAWINGS

12  
13 Figure 1 is a graph depicting the excitation and emission wavelengths  
14 for NADH and Riboflavin;

15 Fig. 2 is a plot of a laser diode optical alignment calibration using  
16 fluorescent latex beads for illustrating a good tight distribution of fluorescence  
17 brightness, as a function of intensity channel, versus number population.

18 Figure 3 is a graph of total fluorescence particles detected using the  
19 laser diode and HeCd lasers sequentially at slow, regular and slow APS flow rates;  
20 the results illustrating substantially continuous and comparable detection results  
21 regardless of the excitation light source;

22 Figure 4 is a graph illustrating the good correlation of fluorescent  
23 particle measurement by the laser diode with live particles as determined with  
24 colonies grown on agar plates, and as sampled from the same aerosol source;

25

1                   Figure 5 is a graph according to a portion Fig. 4, and having an  
2   expanded time scale for the narrow 4 minute window in which the agar plates were  
3   sampled; the arrows illustrating points of coincidence;

4                   Figure 6a, a graph is presented which illustrates the FLAPS2  
5   frequency-tripled, pulsed, solid state YAG laser technology versus the FLAPS1  
6   switched sequentially between use of Nichia 402nm laser diode and the HeCd laser  
7   through a test of introduction of BG spores, slow and regular APS flow rates;

8                   Figure 6b is a graph of the data of Fig. 6a normalized by a factor of  
9   1.567 for slow flow rates;

10                  Figure 7 is a graph of a Spearman rank order, non-parametric  
11   correlation of the data of Fig. 6a illustrating a good correlation coefficient of 0.86;

12                  Figure 8 is a graph of a field measurement test of the laser diode, the  
13   results of which are favorably coincidence with viable counts determined per agar  
14   plates;

15                  Figure 9 and 10 are graphs of another field and comparative  
16   measurement test of the 402nm and the HeCd lasers respectively, both at low  
17   aerosol concentrations while still producing favorably coincidence with viable counts  
18   per agar plates, and at levels above noise;

19                  Figure 11 is a graph which illustrates the laser diode and HeCd laser's  
20   sensitivity respectively to background aerosol material;

21                  Figures 12 and 13 are graphs which illustrate the performance and  
22   capability of the laser diode to sense ovalbumin which was released 200m upwind of  
23   the instrument and the capability of the HeCd laser to sense ovalbumin released  
24   100m upwind of the instrument respectively, and in each case several events met  
25   an alarm threshold when bio-particles were distinguished over background;

Figures 14a and 14b relate to tests performed with a 413nm ion laser of Example 8, and illustrate plots of negative population calibrations of the AF-PMT presented as AF-PMT vs. FS-PMT and AF-PMT intensity;

Figures 15a and 15b are plots of positive population calibrations of the AF-PMT presented as AF-PMT vs. FS-PMT and AF-PMT intensity;

Figures 16a and 16 are histograms of FS-PMT log versus AF-PMT log for the 413nm ion laser at sequentially decreasing power settings of 50, 40, 30, 20, 15, 10 and 5 mW respectively; and

Figures 17a and 17b are comparative plots of the HeCd laser on old and new spores respectively, demonstrating performance which is similar to the 413nm ion laser in the specific environment of the flow cytometer.

# DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

An instrument is provided comprising means for contacting substantially discrete particles of a particle population with an excitation light source, wherein said light source being a laser diode. As set forth in a series of examples, the laser diode has been found to emits a wavelength which is suitable for exciting biomolecules in the contacted particles. While it is anticipated that the laser diode would be implemented as the sole excitation light source in a new instrument, for the purposes of this discussion and as set forthin the examples, a laser diode was conveniently retrofit into an existing prior art FLAPS apparatus so as alternately apply a conventional laser light source or to apply a laser diode source according to the present invention.

As described below, a laser diode emitting a light beam has been tested with particles flowing through a conventional aerodynamic particle sizing

1 (APS) apparatus for a series of Examples 1 – 7 and also through a flow cytometer in  
2 Example 8.

3 Generally, the instrument provides an environment for contacting  
4 subject particles of a population with an excitation laser beam. The prior art FLAPS  
5 apparatus is such a platform suitable for implementing the present invention. A  
6 nozzle is provided for accelerating an airstream containing particles for discharge  
7 past a pair of laser beams. Timing of the particle's flight between laser beams  
8 enables determination of the particle's velocity and size. The arrangement of this  
9 portion of the apparatus is consistent with the conventional APS instrument. The  
10 particles then traverses a laser beam for fluorescence characterization of the  
11 particle to determine biological viability. The details of the APS apparatus and  
12 principles of fluorescence characterization are set forth in detail in US 5,701,012 to  
13 Dr. J. Ho, the entirety of which is included herein by reference. This apparatus  
14 utilizes a HeCd laser and is referred to herein as FLAPS1.

15 A further improved prior art apparatus is disclosed in US 5,895,922 to  
16 Dr. J. Ho, the entirety of which is included herein by reference, and which discloses  
17 a diode-pumped Nd:YLF laser frequency-tripled laser, the system being termed  
18 FLAPS2.

19 The FLAPS2 apparatus further provides a pair of spaced laser beams  
20 for detection of a particle's flight time and size. The excitation laser can be pulsed  
21 to provide selective firing and thus only excite particles matching desired size  
22 characteristics. This feature offers a "smart" technique for interrogating the biological  
23 characteristics of a single particle and also avoids unnecessary firing of the laser at  
24 background material of less than 1  $\mu\text{m}$  in size.

25

1 Limited by their lasers, both FLAPS1 and FLAPS 2 employ an  
2 excitation frequency of between about 325 and 349 nm.

3 Turning to the present invention, a laser diode replaces the cooled  
4 HeCd laser of the FLAPS1 apparatus. Laser diodes are solid state excitation  
5 sources which are small, inexpensive, emit longer wavelengths than the prior  
6 FLAPS apparatus and typically have low power consumption and output. Low cost  
7 visible light laser diodes and their driver circuitry are typically packaged and used in  
8 handheld laser pointers and are button-battery powered. There is no need for  
9 expensive and bulky cooling systems. To Applicant's knowledge, it has not been  
10 suggested to date that this technology is adaptable to biomolecule detection, in part  
11 due to the availability being limited to longer light wavelengths and their low power  
12 output. It was particularly uncertain whether laser diodes could be adapted to the  
13 detection of biological spores, such as anthrax spores, which present a serious  
14 airborne threat.

15 While other apparatus could be applied to contact particles and an  
16 excitation source, a convenient apparatus was available in the FLAPS2 apparatus  
17 described in US 5,895,922, the base instrument comprising an APS having a tubular  
18 nozzle and an airstream containing particles, which may or may not be biological,  
19 and may or may not be viable. The APS acts as a sequencer, directing the particles  
20 to exit the nozzle in a sequential manner, traveling downwardly along a linear flight  
21 path and traverse two path-intersecting laser beams. Preferably, two laser beams  
22 are used for particle detection and time-of-flight determination. The position of each  
23 particle in the airstream is determined as a function of time and the particle's time of  
24 flight is measured between two points along a flight linear path so as to establish the  
25 particle's size

1 For the purposes of the present invention, the apparatus supporting  
2 the 340-360 nm HeCd laser of FLAPS1, its power supply and optics were retrofitted  
3 with a laser diode emitting a nominal 402-405 nm laser beam. For comparison  
4 purposes, the FLAPS1 apparatus was modified to enable alternate use of either the  
5 HeCd or the laser diode.

6 A suitable laser diode is a Nichia light source which emits at about 402  
7 nm. Such a light source is a Nichia laser diode, model number NLHV500A (nominal  
8 8 mW) by Nichia Corporation, Tokushima, JAPAN. A similar wavelength (405nm),  
9 higher power diode is the 30 mW model NLHV3000E. Other laser diodes are  
10 available from the same manufacturer which range in output from 400 to about 450  
11 nm, with others being developed. Common laser pointers utilize diodes capable of  
12 wavelengths typically in the range of 645-680nm.

13 The excitation source is positioned just below the nozzle and below the  
14 APS laser beams. Laser beam output is aligned by a collimating lens. A suitable  
15 lens is a model 307-4606-670, 4.6mm focal length, numerical aperture 0.53  
16 available from Optima Precision, Inc., West Linn, OR.

17 The particles are preferably first sized to determine respirability. The  
18 particles are then excited by either the HeCd or the laser diode laser beam and the  
19 particles are then monitored for fluorescent emission from biomolecules which is  
20 characteristic of viable bioparticles. Fluorescence is detected using a using a  
21 photon counter such as a PMT. As set forth in the Ho patents, a microprocessor  
22 compared each contacted particle's fluorescent intensity signal against  
23 predetermined criteria for establishing whether that particle is a biologically viable  
24 particle or an inert particle.

25

1 As shown in Fig. 1, the known biomolecule NAD(P)H exhibits peak  
2 excitation at wavelengths of approximately 325nm – 354nm with characteristic  
3 emission between 420nm and 560nm and with a peak at approximately 460nm.  
4 Riboflavin, on the other hand, exhibits peak excitation at approximately 400nm, with  
5 characteristic emission between 475nm and 580nm. Whether riboflavin exists in  
6 biomolecules had not been determined. This data represents material published by  
7 Li et al. in *Monitoring Cell Concentration and Activity by Multiple Excitation*  
8 *Fluorometry*, Biotechnol. Prog., 1991,p:21-27. The prior art system using the HeCd  
9 laser was designed to illuminate the particles at 325nm and detect fluorescence in  
10 the region from 420nm to 560nm.

11 The present system, using the Nichia laser diode, was applied to  
12 ascertain if the low power, higher frequency laser diode could excite particles at  
13 402nm and detect fluorescence as effectively as the higher power, complex and  
14 expensive HeCd laser implementation. Heretofore, applicant was unaware of  
15 research which confirmed the presence of riboflavin in the nearly inert spore form.  
16 Solovieva et al (Solovieva, I.M., R.A. Kreneva, D.J. Leak and D.A. Perumov. 1999.  
17 *The ribR gene encodes a monofunctional riboflavin kinase which is involved in*  
18 *regulation of the Bacillus subtilis riboflavin operon*. MICROBIOLOGY-UK, 145: 67-  
19 73.), however, reported isolation of the *Bacillus subtilis* DNA that contained the  
20 riboflavin operon and was able to clone the operon into *E.coli*, suggesting the ability  
21 of *Bacillus subtilis* to synthesize riboflavin. It was uncertain as to whether levels, if  
22 present, were sufficient to allow detection.

23 The following examples illustrate the surprising ability of the  
24 economical low power, longer wavelength laser diode to successfully excite and  
25 fluoresce some form of biomolecule, other than the known NADH, in viable particles.

1 The applicant hypothesizes that one of the biomolecules which is fluorescing at  
2 these longer wavelengths is riboflavin.

3

4 EXAMPLES

5 Unless otherwise stated for each individual example, the following  
6 equipment and conditions were used for comparing the performance of prior art:  
7 FLAPS1 using the HeCd laser, the prior art FLAPS2 using a pulsed solid state  
8 Nd:YLF laser, and the FLAPS1 particle apparatus in combination with the present  
9 invention's laser diode. Tests were conducted to determine whether biologically  
10 viable particles could be distinguished from a particle population containing a  
11 mixture of biologically viable and biologically inert particles.

12 As suggested above, for comparative experimentation purposes only,  
13 a FLAPS1 instrument was constructed having both a HeCd laser and a Nichia laser  
14 diode installed for ease of comparison of the individual results of excitation. In  
15 incorporating both prior art gas laser and diode lasers into the same instrument, it  
16 became necessary to prevent the excitation light from interfering with the  
17 fluorescence detector. Therefore, a corresponding long pass filter, having a cut off  
18 at 435nm, was provided in addition to other existing filters for the FLAPS1 when the  
19 Nichia laser diode was used. Such a filter is a Schott Glass GG 435, available from  
20 Melles Griot, Irvine, CA . The GG435 filter was placed between the primary light  
21 collection lens and the dichroic filter.

22 To enable alternate selection of the light sources in the following  
23 experiments, a steering mirror on a Kinematic mirror mount was inserted along the  
24 exciting beam path. Such a mirror and Kinematic mount are a 02 MFG 015/038  
25 mirror available from Melles Griot, Irvine, CA and a BKL-4 mount available from

1 Newport Corporation, Irvine, CA.

2           The laser diode was operated in constant current mode, with a power  
3 readout connected to the laser's internal photodiode. The power tended to droop as  
4 the laser warmed over the course of each trial. Accordingly, during initial startups,  
5 manual adjustments were made to maintain laser diode output power between 12  
6 and 14 mW. It was noted that power droop and compensating manual adjustments  
7 could affect data displays as slight distortions in the fluorescence intensity data.

8           In the case of the FLAPS2 apparatus, to compensate for higher than  
9 optimal pulse energy, an optical density filter was used to pass only 10% of the laser  
10 beam output to contact the particle. This resulted in lower background noise and  
11 better signal-to-noise characteristics. The laser was triggered when a particle was  
12 detected with a minimum time between triggers of 200  $\mu$ sec. To compensate for  
13 different particle flight times due to particle size, a "skeet shooting" look-up table was  
14 used to synchronize laser trigger times. The table provided information that relates  
15 particle size to time delay for firing the laser. Accordingly, through firmware  
16 commands, selective firing could be exercised to excite only particles of desired size  
17 characteristics, avoiding unnecessary firing to contact mere background particles.

18           During real time sampling, the instrument presents size and  
19 fluorescence intensity information for each contacted particle. Data derived from a  
20 sampling period, typically 3 seconds, can be reduced to a fractional number (gated  
21 % fluorescent); this represents the percent of particles that exhibited fluorescent  
22 signal above a preset size and intensity threshold. A threshold level can be  
23 determined for each environmental condition by observing the prevailing background  
24 conditions. An alternate method to present fluorescence data is to derive a "signal-  
25 to-noise" ratio. This is done by comparing the mean of background gated %

1 fluorescent with new “unknown” or “biological” data.

2           Note that a prototype aerosol concentrator (model XMX, SCP  
3 Engineering, St. Paul, MN) was used as a front end to the FLAPS2 intake to enrich  
4 the population of background fluorescent particles for better statistical counting.  
5 The concentrator was optimized to minimize size, weight and power consumption;  
6 operating at 400 liter/min and concentrating to 1 liter/min delivered to the FLAPS2  
7 intake. Improved particle throughput provided by this setup facilitated a rapid  
8 sampling time of 3 seconds. In practice, an additional 1 second penalty was incurred  
9 for computational and data handling overhead. By this protocol, aerosol data could  
10 be collected continuously every 4 seconds over long periods unattended by the  
11 operator.

12           The instrument was calibrated for use at a wavelength of 402 nm using  
13 an aerosol of fluorescent latex beads (catalog number B0200, Duke Scientific  
14 Corporation, Palo Alto, CA). The latex beads exhibit a blue fluorescent dye having  
15 three excitation peaks: 365nm, 388nm, and 412nm. The 365nm and 388nm  
16 excitation emit at a peak at 445nm and the 412nm excitation emit at a peak of  
17 445nm, with a secondary emission peak at 473nm. As 402nm wavelength falls on  
18 the sides of both the 388nm and 412nm excitation peaks, the result is a combination  
19 of the 445nm and 473nm emission peaks. The latex beads were aerosolized using  
20 a TSI Model 3076 nebulizer, together with a TSI 3074B air supply filter and a TSI  
21 3012 aerosol neutralizer.

22           Biological aerosol dissemination of *Bacillus subtilis* var *niger* spores  
23 (BG spores) was accomplished with a Hudson nebulizer (Model 1700, Hudson  
24 Oxygen Therapy Sales Co., Wadsworth, OH) at 172-206 kPa (25-30 psi). A  
25 suspension containing BG spores in the range of 5 to 30 µg plus 4 mg silica gel

1 (Syloid 245, Davison Chemical, Baltimore, MD) per ml was used as the starting  
2 material.

3 Field biological aerosol dissemination of BG spores took place about  
4 100m to 250 m upwind of the detection system. Culturable particles were impacted  
5 into an agar plate and grown to determine the presence of live content.

6 Egg albumin powder (A5253) used as a protein-based toxin simulant,  
7 was obtained from Sigma Chemical Co. An aerosol was produced by a Nordson  
8 Tribomatic® 500 spray gun (Canwest Pumping Systems Ltd. Calgary, Alberta  
9 Canada).

10 A correlation analysis was performed to estimate the degree to which  
11 FLAPS measurements vary together with live reference data and the common  
12 cause is the presence of live biological aerosols. The data was tested for distribution  
13 normality using non-parametric methods

14 In the following Examples 1 – 6, a nominal 402-405 nm laser diode  
15 was used to confirm the detection of biomolecules. In Example 8, recognizing that  
16 that BG spores may also be sensitive to even longer wavelengths, a further  
17 conventional ion laser was also applied, specifically at 413 nm.

18

#### 19 Example 1

20 As a calibration check for good optical alignment, standard fluorescent  
21 latex beads were examined with FLAPS1 using the Nichia laser diode light source.  
22 Having reference to Fig. 2, the data is seen to conform well to a Gaussian  
23 distribution, with a fit correlation coefficient of 0.96. As measured by the size  
24 distribution capability of the instrument, 2 µm beads were occupying 2 size  
25 channels, indicating tight banding of the sizing function. The conclusion was that the

1 optical components were properly installed and optimized for fluorescent detection  
2 of particulate aerosols.

3

#### 4 Example 2

5 As shown in Fig. 3, background measurements were performed in the  
6 FLAPS1 particle chamber followed by the gradual introduction of a spore aerosol  
7 (BG). The experiment was started with a slow sample flow rate to improve the signal  
8 to noise measurement due to slower particle transit time in the laser diode beam.  
9 Fluorescence signals remained relatively constant even when the Nichia laser was  
10 turned on (see time 14:39:25) suggesting that HEPA filtered air in the chamber  
11 contained little, if any, background material that had fluorescence characteristics.  
12 The aerosol spray of biological aerosols was introduced at 14:40:32 and shortly  
13 thereafter, at 14:41:13, the first visible BG fluorescence was measured.

14 Sample flow rate was increased to a regular flow rate at 14:42:37,  
15 resulting in some slight effect on the rate of detection, as observed from the slope of  
16 the trace. The measurement rate continued to rise until at 14:44:30 when the 435nm  
17 filter was removed in preparation for changing from the Nichia laser to the HeCd  
18 laser. A dip in the measurement can be observed as the laser was changed  
19 (14:45:00). Following a switch to the HeCd laser, the data resumed a similar rate of  
20 increase as had been observed with the Nichia laser. Moreover, when the flow rate  
21 was attenuated to the same slower level as applied at the beginning of the  
22 experiment, the fluorescent particle sampling rate appeared to drop significantly,  
23 similar to the measurements at the start of the test.

24

1    Example 3

2                    Having reference to Fig. 4, in another BG aerosol chamber test using  
3    the FLAPS1 particle chamber, the fluorescent particle measurement was correlated  
4    for a 4 minute collection span with colony-forming live particles. Slit sampling was  
5    performed to capture live particles in high resolution with respect to time, to resolve  
6    particulate material in 1 second time slices, over a 4 minute time span. The aerosol  
7    generator was a spinning disk device designed to produce relatively high  
8    concentration of particles. The results of the live particles were expressed as Agent  
9    Containing Particles per Liter of Air (ACPLA).

10                  The rate of increase of fluorescence using the Nichia laser, roughly  
11    paralleled that of the live particles.

12                  The same reference data is shown in Fig. 5, expanding the 4 minute  
13    collection span so as to better inspect and compare the dynamics of the two  
14    measurement methodologies, the laser method being instantaneous, the agar plate  
15    method being long term (and clearly ineffective to warn of a biological threat). The  
16    arrows in Fig. 5 provide subjective and favorable indications as to when the two data  
17    sets appear to coincide.

18

19    Example 4

20                  A FLAPS2 instrument powered by a frequency-tripled Nd:YAG light  
21    source was used to measure BG aerosol simultaneously with the experimental  
22    FLAPS1 instrument equipped with interchangeable Nichia laser diode and the HeCd  
23    laser light sources. A similar experimental approach as used in Example 3, was  
24    used to supply a dynamic BG aerosol concentration in a chamber while optical  
25    measurements were recorded. The response of the two FLAPS instruments was

1 compared.

2 As shown in Fig. 6a, the Nichia laser diode was selected in FLAPS1  
3 and the aerosol generator was run at a slow flow rate at the beginning of the test.  
4 Shortly after the aerosol spray started, both FLAPS1 and FLAPS2 detectors  
5 measured the presence of fluorescent particles. The absolute counts were  
6 dissimilar, as the FLAPS2 was a pulsed device, providing a more energetic  
7 excitation for each contacted particle, resulting in emission signals from small or dim  
8 particles. No attempts were made to detune the FLAPS 2 light source.

9 FLAPS1 was switched to run on a regular sampling flow rate at  
10 11:24:00 and again using the Nichia laser diode. The two FLAPS apparatus  
11 registered roughly similar particle sampling rates. FLAPS1 was then switched to the  
12 HeCd laser at about the same time the aerosol was stopped. Subsequent HeCd  
13 data roughly paralleled the decreasing fluorescence measured by FLAPS2.

14 Throughout the testing, all three lasers induced fluorescent signals that  
15 reflected the dynamic presence of BG spore particles in the chamber.

16 Having reference again to Fig. 6a, FLAPS1 data appears disjointed  
17 due to switching between slow and regular flow rates giving the appearance of  
18 slightly different particle counts rates under each flow regime. In contrast, the  
19 FLAPS2 curve is a smooth trace disrupted only by time gaps reflecting changes in  
20 experimental conditions. Mathematically, compensation was attempted for the  
21 different flow rate by normalizing the particle counts. In Fig. 6b, slow flow values  
22 were adjusted by a factor of 1.567 to account for the flow differential.

23 Further, FLAPS data can be presented as a fraction of particles  
24 greater than a particle size cut, expressed as percentage of total particles  
25 measured. This better reflects biological content in environmental conditions where

1 absolute particles may fluctuate over time but the size distribution is expected to  
2 remain fairly constant in the absence of unnatural disturbances.

3 Referring to Fig. 6b, data is plotted as gated % fluorescence,  
4 illustrating a different dynamic expression of the aerosol conditions in the chamber.  
5 The gated fraction represents particles  $>2\ \mu\text{m}$  as a percentage of the total particles  
6 at a given time, measuring spore aggregates rather than individuals. Further, the  
7 gated % fluorescent value prior to the aerosol was highly variable, due to low count  
8 statistics. A dramatic increase in the gated fraction was observed once the aerosol  
9 was started. The drop in this value over extended time reflects changes in particle  
10 size dynamics. For example, the larger population may have been reduced due to  
11 falling out more rapidly with respect to the smaller particles. The restoration to  
12 regular flow also affected the population balance of the larger size fraction.

13 Referring to Fig. 7, since the data sets were not normally distributed,  
14 non-parametric statistical methods were used to derive a correlation coefficient  
15 (Spearman rank order). A correlation coefficient of 0.86 was obtained, suggesting a  
16 good correlation between the fluorescent particles detected by FLAPS1 and  
17 FLAPS2, and that the two instruments were measuring the same aerosol cloud in  
18 the test chamber and tracking its concentration changes with similar fidelity. Since  
19 the laser diode was selected in FLAPS1 while the YAG laser in FLAPS2 was also  
20 operating, this correlation suggested that both types of lasers detected the same  
21 aerosol cloud. Moreover, during this process, the change in laser in the FLAPS1  
22 apparatus further illustrates that the use of the Nichia laser diode or the HeCd laser  
23 were equally effective and exerted no significant effect in the measurements.

24  
25

1 Example 5

2  
3 Previous experiments were performed in an aerosol chamber, which  
4 allow controlled studies to be performed reproducibly under similar specified  
5 conditions. Biological aerosol detection, however, must perform acceptably in the  
6 natural environment under unpredictable conditions. In order to test the system's  
7 ability to function in the natural environment, the FLAPS1 instrument was installed in  
8 a trailer located in the field. The air intake to the instrument was about 3 meters  
9 above the ground.

10 Reference samplers (slit samplers) were positioned about 10 meters  
11 away, also about 3m above ground level. Although not ideal, as the distance  
12 between the systems could contribute to timing errors in tracking dynamic aerosol  
13 activity, this set up, under fair weather conditions, has been previously shown to  
14 provide acceptable results.

15 As set forth in Fig. 8, in the presence of a high concentration of BG  
16 spore aerosol, visual inspection of the fluorescence signals using the Nichia laser  
17 diode (gated % FL) suggests the signals correlate well with viable counts, expressed  
18 again as Agent Containing Particles per Liter of Air (ACPLA).

19 As shown In Fig. 9, repeat testing of the Nichia laser on a second day  
20 using low aerosol concentrations (6-10 ACPLA), yielded measurements discernable  
21 from background, as did subsequent comparative testing using the HeCd laser as  
22 shown in Fig. 10.

## Example 6

In practice, a biological detector also measures material that occurs in the background. In the rare instance when an unusual biological cloud appears, the instrument must be capable of registering a discernable difference between the background and the biological cloud. Hence, the background measuring performance of an instrument must be carefully characterized.

As shown in Fig. 11, measurement of background aerosol was conducted using the FLAPS1 apparatus fitted with a pre-concentration virtual impactor to enhance particle counts presented to its intake. Using the Nichia laser, and over a 4.5 hour sampling period, the fractional background fluorescent population showed only gradual changes. Occasional spikes occurred but were only of short duration.

Similarly, for the latter part of the measurements and switching to the HeCd laser yielded comparable results. The slightly lower fractional levels in the latter trace may be a function of the laser power (15 versus 12 mW) although this has not been verified. Nevertheless, the two measurement systems were within acceptable performance levels, given the influence of environmental variables.

## Example 7

Having reference to Figs. 12 and 13, biomolecule simulant response trials were performed. Ovalbumin (OV) available from Sigma Chemical Co. was used as a simulant for protein based toxins. In its pure form only short light stimulation at 260-280nm produces fluorescence from aromatic amino acids present in ovalbumin, per Groves, W.E., F.C. Davies and B.H. Sells. 1968. *Spectrophotometric determination of microgram quantities of protein without nucleic*

acid interference. Anal. Biochem 22:195-210. However, as reported in Boulet, C.A., J. Ho, L. Stadnyk, H.G. Thompson, M.R. Spence, G.A. Luoma, R.E. Elaine and W.E. Lee. 1996. *Report on the Canadian Integrated Biological Detection System Trial Results of the Combined Joint Field Trials II, for Biological Detection. Defence Research Establishment Suffield SR 652 (Unclassified)*, in previous field trials with OV aerosol using this specific product, excitation with UV light at 340-360nm produced measurable fluorescence. The material used had a yellowish tinge, on visual inspection, suggesting the presence of egg yolk contaminants which resulted in the fluorescence detected.

Having reference to Fig. 12, OV aerosol was detected using the Nichia laser, producing comparable results and alarm signals to those obtained using the HeCd laser, as shown in Fig. 13. Background noise levels in the test for Fig. 12 were higher than that experienced for Fig. 13 and were most likely due to environmental conditions on the day of testing. Reference data to verify the presence of OV was not available for comparison. Current methodology for collecting OV aerosol utilizing a glass impinger with a time resolution of 5 minutes would have been unsuitable for comparison with FLAPS1 data, the latter having time resolution of 4 seconds.

## CONCLUSIONS

The Nichia laser, without the cost, nor the power and technical support requirements of the more conventional gas HeCd or diode pumped solid state YAG lasers, performed equally well. The particular tested laser diode had a wavelength longer than Applicant's experience and thus the results were doubly surprising; first having proved that a low power laser diode can detect biomolecules, and secondly

1 that biomolecules could be excited in particles of interest, even in spores, which  
2 could be excited at a larger and longer range of wavelengths than previously thought  
3 practical.

4 FLAPS1 performance using both the HeCd laser or the Nichia laser  
5 diode gave well correlated performance characteristics when presented both with  
6 biological aerosol simulants OV and with BG spore aerosol. Spores, by their  
7 physiological nature, contain very little biological material and the individual units are  
8 no larger than 0.7  $\mu\text{m}$  and are enclosed by a very refractile spore coat. Due to this  
9 refractile characteristic, conventional light microscopy of spores reveal very little of  
10 its cellular content. In contrast, vegetative cells are better subjects for examination in  
11 that they can easily be stained. Thus, performance of biological detectors must be  
12 rated by their ability to measure spore aerosols and in these experiments and it has  
13 been shown that 10 ACPLA could be detected (Fig. 9).

14 In the original FLAPS1 design, where HeCd 340-360nm excitation light  
15 was used, it was speculated that the fluorescing molecules were most probably  
16 NADH, as shown in Fig. 1. The Nichia laser diode light source emits at about 402  
17 nm, making it possible that the excited molecule is riboflavin, as also illustrated in  
18 Fig. 1. In addition, it can be seen that at this wavelength, a small portion at the  
19 upper end of NADH excitation spectrum may make a small contribution to the total  
20 fluorescent signals. To applicant's knowledge, measurement of riboflavin in spores  
21 has not been reported so it is impossible to verify this claim. However, in the  
22 literature, autofluorescence from blue and near UV excitation has been reported for  
23 a brackish water ciliate that feeds on cyanobacteria (Selbach, M and H.W.  
24 Kuhlmann. 1999. *Structure, fluorescent properties and proposed function in*  
25 *phototaxis of the stigma apparatus in the ciliate Chlamydomonas mnemosyne*. J. Expl.

1 Biol. 202:919-927.). Also Van Schaik, H.J., C. Alkemade, W. Swart and J.A. Van  
2 Best. 1999. *Autofluorescence of the diabetic and healthy human cornea in vivo at*  
3 *different excitation wave-lengths*. Expl Eye Res. 68:1-8 attributed 405 nm excited  
4 fluorescence in cornea of diabetes mellitus patients to flavins. Finally, as stated  
5 earlier, Solovieva et al. reported isolation of the *Bacillus subtilis* DNA that contained  
6 the riboflavin operon and was able to clone in to *E. coli*. At least this suggests that  
7 BG has the capability to synthesize riboflavin. Whether the individual spore contains  
8 significant amounts of the material is difficult to predict.

9 Whether the low power laser diodes can detect biomolecules in  
10 particles of interest is now proved. Further, laser diodes are becoming available  
11 across a larger range of wavelengths. While not all wavelengths are suitable for  
12 stimulation of biomolecules, the above examples have demonstrated that the known  
13 range should be expanded. Accordingly, it is seen that laser diodes can also be  
14 operable at longer wavelengths. The following example illustrates a further and  
15 longer wavelength suitable for application when such a laser diode is economically  
16 available.

#### 17 Example 8

19 An analysis of bacterial stimulation and fluorescence was also  
20 conducted at higher wavelengths to further expand the known range of fluorescence  
21 of biomolecules, whether or not the biomolecule responsible for the fluorescence is  
22 known. An ion laser, model Coherent Enterprise II (Coherent, Inc. of Santa Clara,  
23 CA, USA), was used which produced a wavelength of about 413 nm. Note that the  
24 characteristics of the Enterprise II are particularly demonstrative of the  
25 disadvantages and of the need for a practical laser diode apparatus. The Enterprise

1 II laser's plasma tube has a suggested life of about 5000 hours and at has a 220 V,  
2 single phase maximum power requirement of about 7 kVA. Further, note that the  
3 manufacturer, Coherent, Inc., suggests that there are several options are available  
4 for keeping the Enterprise II cool for operating at peak performance. About 5 kW of  
5 waste heat from the system can be removed with water-to-air or water-to-water heat  
6 exchangers, with chillers, or by direct cooling with water that meets our published  
7 quality guidelines. The Enterprise II can optionally be cooled with a water-to-air heat  
8 exchanger when sufficient water is not available, water quality is poor, or air-cooled  
9 operation is desirable. This cooling unit can be located up to 15 meters away and be  
10 operated remotely through the power supply control panel.

11 In the example, particles were contacted and exposed to the ion  
12 laser's beam using a Beckman Coulter ELITE™ flow cytometer. For comparison,  
13 the flow cytometer was also fitted with a HeCd laser emitting 352 nm for applying  
14 the known excitation and response of NADH. The fluidic conditions of the flow  
15 cytometer were optimized for smaller particles such as bacteria as the smaller the  
16 particle the less they follow the hydrodynamic focusing principle. To maximize the  
17 time the particles spend in the sensing area (at the flow cell), the volume flow rate  
18 settings were lowered on the flow cytometer. The optimal sheath pressure for  
19 acquiring the BG spores was found to be 8.0 psi, with a corresponding sample  
20 pressure of approximately 7.2-7.4 psi (based on a 413nm laser output of 50 mW as  
21 the required sample pressure was later found to be dependent on laser power  
22 output). Serial dilutions of BG spores were prepared in pure water to yield  
23 concentrations of  $10^3$  -  $10^9$  /mL. The dilutions were run on the cytometer using the  
24 HeCd laser at 351 nm to determine the optimal concentration of BG spores for  
25 further experiments. At sheath and sample pressures of 8.0 and 7.2 psi,

1 respectively, the 106/mL concentration yielded the most desirable rate in  
2 events/second for data collection.

3 One of the main objectives of the study was to measure and compare  
4 the intrinsic fluorescence of BG spores using the HeCd (352 nm) and the 413 nm  
5 light sources. Given that each of the lasers emitted at a different wavelengths, it was  
6 necessary to select a filter configuration suitable for the laser light plan and the  
7 fluorescence of interest. Accordingly, different filter configurations were used to  
8 properly measure the intrinsic fluorescence of BG spores excited by the respective  
9 lasers. For the 352 laser, a blocking bandpass filter at 450 nm and a dichroic filter  
10 of 505 nm were provided to ensure that excitation light scatter or other artifacts were  
11 not detected by the photomultiplier tube (PMT) used to detect emissions in the  
12 anticipated range of about 410 – 540 nm. Similarly, for the 413 laser, a blocking  
13 bandpass at 440 nm was provided to ensure only that the PMT only detected  
14 fluorescence emissions which were in the anticipated estimated range of 480 – 570  
15 nm.

16 To determine power and fluorescence resolution, a background or  
17 negative population was calibrated. As shown in Figs, 14a and 14b, in a log-log plot  
18 of auto-fluorescence PMT (AF-PMT) output vs. forward scatter PMT (FS-PMT), the  
19 AF-PMT was adjusted so that a non-fluorescing 1 $\mu$ m beads were detected in the  
20 first decade of the log scale (about 0.1-30). As shown in Fig. 15a and 15b, a  
21 positive population of 10 $\mu$ m fluorescing beads was checked to ensure AF-PMT  
22 response across all four log output decades (0.1 – 1000).

23 After background fluorescence levels were run and PMT voltage levels  
24 were set, the BG spores were run through the cytometer for the 413 laser and for  
25 the 352 HeCd laser. The HeCd had a power output of about 30mW and the 413 nm

1 laser was run at power settings ranging from about 3 – 50 mW.

2 As a measure of BG spore auto-fluorescence, resolution forward  
3 scatter was investigated. Using the 413 nm laser, diminishing power levels were  
4 applied. Having reference to Figures 16a – 16g, the resolution of auto-fluorescence  
5 was optimal at a power setting of 10 mW. In Figs. 16a – 16e and 16g, higher and  
6 lower power settings of 50-15 mW and 5 mW did not produce the same levels of  
7 light scattering from BG spores. Light events diminished significantly at power levels  
8 below 10 mW. Accordingly, using the cytometer environment, the minimum 413nm  
9 laser power output for obtaining usable fluorescence emission was determined to be  
10 about 10mW (Fig. 16f). The results were similar under repeats tests where new or  
11 old spores were used and whether the experiments applied increasing power  
12 through 10 mW or reducing power.

13 For confirmation of the viability detection results and comparison of the  
14 light sources applied in the same flow cytometer environment, tests were also  
15 conducted with the known NADH biomolecule detecting HeCd laser. Negative and  
16 positive populations were applied and the AF-PMT adjusted. With the HeCd laser,  
17 instrument noise is prevalent. The FS-PMT peak signal was selected as the  
18 discriminating parameter and set accordingly to reduce much of the instrument  
19 noise. As shown in Figs. 17a and 17b, and at HeCd laser power of 30 mW, the prior  
20 art laser was similarly able to discern sufficient events to detect auto-fluorescence.

21

## 22 Summary

23 In summary, we have shown that by using a small, low-power laser  
24 diode that emits at 402-405 nm, it is possible to replicate the viability detection  
25 performance of the 340-360 nm light sources. Further, having confirmed that

1 fluorescence is detectable at even longer wavelengths (having further applied a  
2 conventional 413 nm laser source) then as additional wavelengths of laser diodes  
3 become available, they can also be applied in a superior and economical biological  
4 detection apparatus.

5               The findings from this study have critical implications for the future of  
6 biological detection, both for military applications as well as in environmental  
7 monitoring. With this new inexpensive and compact light source, a detector requires  
8 no active cooling system, consumes little power and weighs much less than current  
9 instruments. The associated cost reduction not only benefits large industrial and  
10 defence applications but also make it possible for commercial and home use.

11              It is understood that the above description represents the preferred  
12 embodiment and that other embodiments are possible including combinations with  
13 flow cytometers, cloud chambers and other such apparatus for contacting the  
14 particles and the excitation lasers.

15